L-Type calcium channel blockers modulate the microvascular hyperpermeability induced by platelet-activating factor in vivo

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Purpose: **Platelet-activating factor (PAF) is a potent phospholipid mediator of the microvascular dysfunction associated with ischemia-reperfusion injury. Because changes in cytosolic-free Ca^{2+} concentration are essential in PAF cellular signaling, we formulated the hypothesis that blockade of Ca^{2+} entry may inhibit the PAF-induced microvascular dysfunction.**

Methods: **To investigate this hypothesis two L-type calcium channel blockers, verapamil and nifedipine, were applied to the hamster cheek pouch before the topical PAF challenge was undertaken. Permeability was assessed by measurement of the plasma clearance of fluorescein isothiocyanate dextran, 150,000 mol wt. The arteriolar diameter was measured simultaneously to evaluate the effects of L-type calcium channel blockers on PAF-induced vasoconstriction.**

Results: **Baseline clearance was 498.7 ± 225.0 nl/60 min/gm (mean ± SE). PAF at 10^{-8} mol/L ($n = 5$) increased clearance to 3753.8 ± 572.8 nl/60 min/gm ($p < 0.01$). Pretreatment with verapamil (2 mg/kg; $n = 5$) significantly reduced the increase in permeability caused by 10^{-8} mol/L PAF (1909.1 ± 620.2 nl/60 min/gm; $p < 0.05$). Nifedipine ($5 - 10^{-6}$ mol/L; $n = 5$) also significantly attenuated the impact of 10^{-8} mol/L PAF (2037.2 ± 427.5 nl/60 min/gm; $p < 0.05$). Neither verapamil nor nifedipine affected PAF-induced vasoconstriction.**

Conclusion: **The significant inhibition of the increase in permeability by the L-type calcium channel blockers suggests that these compounds may be useful in the management of PAF-induced hyperpermeability. (J VASC SURG 1995;22:732-41.)**

Platelet-activating factor (1-O-alkyl-2-O-acetyl-*sn*-3-phosphorylcholine; PAF) is a potent phospholipid mediator involved in a variety of inflammatory and allergic reactions, including anaphylaxis, endotoxin shock, gastrointestinal ulceration, and ischemia-reperfusion (IR).[1-3] PAF is produced in response to hypoxia and increases adherence properties of cultured porcine aortic endothelial cells[4] and human umbilical vein endothelial cells.[5] In vivo, PAF induces macromolecular leakage and arteriolar constriction and adhesion of leukocytes[6-8] and mediates leukocyte–endothelial cell interactions in IR.[9-11] These data suggest that PAF plays an important role in the pathogenesis of IR injury.

PAF is activated by way of multiple signaling pathways through specific receptors on the plasma membrane.[1,12,13] These signaling mechanisms lead to an increase in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting both from intracellular Ca^{2+} release mediated by inositol 1,4,5-triphosphate (IP_3) through phospholipase C (PLC) activation and from Ca^{2+} influx.[2,12] However, the in vivo actions of PAF-induced Ca^{2+} entry into cells have not been elucidated entirely. In this study we investigated the

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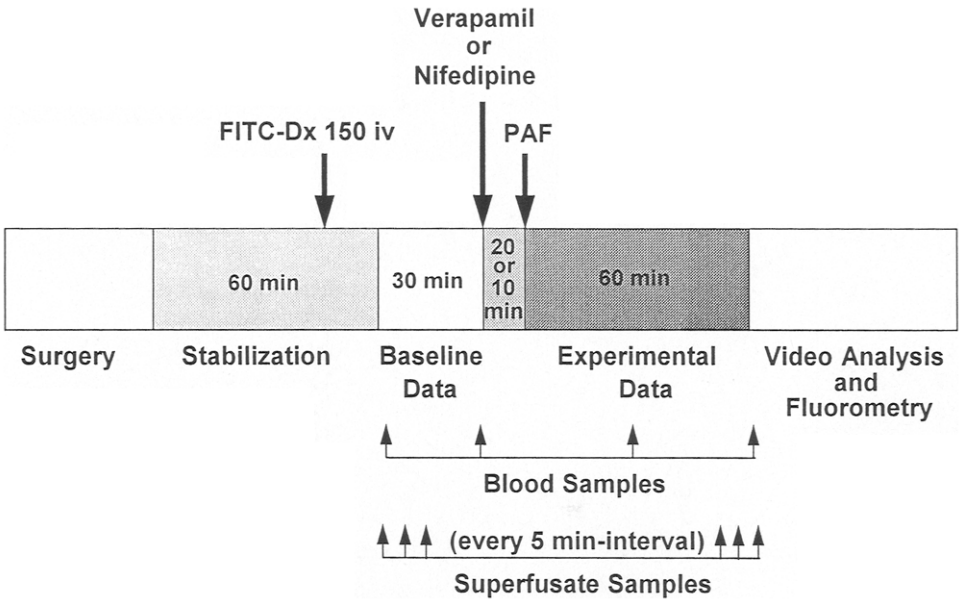

Fig. 1. Diagram of experimental protocols. Verapamil (2 mg/kg) was administered intravenously over 10-minute period. PAF challenge was instituted 10 minutes after stopping application of verapamil. Thus 20-minute period refers only to sum of time of administration of verapamil plus time allowed for recovery of blood pressure. Nifedipine at 10^{-7} mol/L or 5×10^{-6} mol/L was applied topically to hamster cheek pouch for 10 minutes. PAF challenge was implemented immediately after application of nifedipine.

effect of calcium channel blockers on the hamster cheek pouch as an in vivo experimental model for the role of calcium entry in the microvascular changes induced by PAF. We applied two L-type voltage-operated channel blockers—verapamil (phenylalkylamine) and nifedipine (a 1,4-dihydropyridine [DHP] derivative)—to evaluate the effect of PAF-induced calcium entry on microvascular function.

MATERIAL AND METHODS

Animal preparation. Thirty-seven male golden Syrian hamsters (weighing 80 to 120 gm) were anesthetized with sodium pentobarbital (60 mg/kg, administered intraperitoneally). Tracheotomy was performed to ensure clear airway passages. The animal was placed on a heating pad to keep the body temperature at 37° C throughout the experiment. The right jugular vein was cannulated for the administration of supplemental doses of anesthetic, macromolecular tracer, and verapamil. The right carotid artery was cannulated for collection of blood samples and for monitoring of systemic blood pressure. The left hamster cheek pouch was prepared for intravital microscopic observation and intervention as described previously.[7,14] Briefly, the cheek pouch was carefully exposed by removal of the connective tissue. A two-piece acrylic resin chamber (1 ml of reservoir capacity) was attached to the cheek pouch. The cheek pouch was continuously superfused with bicarbonate buffer solution (NaCl, 131.9 mmol/L; KCl, 4.7 mmol/L; $CaCl_2$, 2 mmol/L; $MgSO_4$, 1.2 mmol/L; and $NaHCO_3$, 18 mmol/L) at a rate of 1 ml/min. The buffer solution was adjusted to pH 7.35, warmed to 35° C, and equilibrated with 95% nitrogen/5% carbon dioxide. The cheek pouch preparation was allowed to stabilize for a 1-hour period before any control measurements were performed.

Experimental protocols. The protocols are illustrated in Fig. 1. The direct effects of the vehicle on the microvascular network, if any, were tested in three animals for the entire duration of the experimental protocols. In agreement with our previous experience, the vehicle had no effect on microvascular dynamics.[6,7] After the baseline data were collected, PAF at 10^{-8} mol/L ($n = 5$), or 10^{-7} mol/L ($n = 6$), was topically applied to the cheek pouch for 3 minutes by means of temporary interruption of the superfusate flow. Each animal received only one dose of PAF. After PAF challenge, the superfusate flow was reestablished and the effluent was collected at 5-minute intervals for 1 hour. In nine animals, verapamil (2 mg/kg) was applied intravenously over

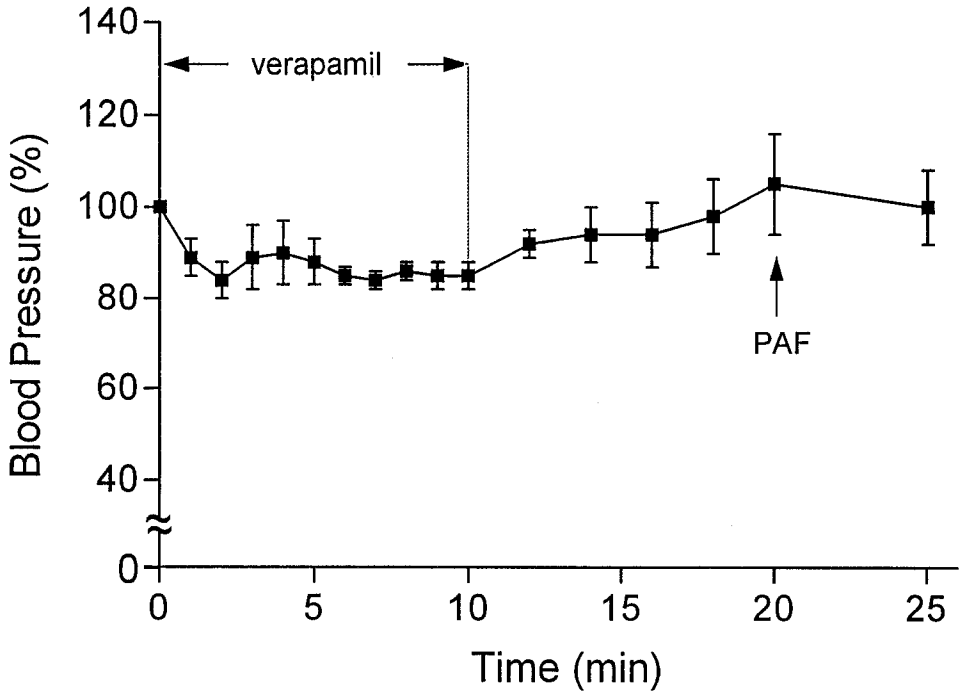

Fig. 2. Effect of verapamil (2 mg/kg administered intravenously) on systemic blood pressure. Ordinate shows percent change in blood pressure normalized to control. Blood pressure returned to 100% of control within 10 minutes. Data are presented as mean ± SE (n = 4).

10 minutes. Because this dose of verapamil caused approximately 15% decrease in systemic blood pressure during its application, PAF challenge was instituted on return to baseline values of systemic blood pressure. Blood pressure returned to baseline values usually 10 minutes after stopping the application of verapamil (Fig. 2, n = 4). In 10 animals, nifedipine at 10^{-7} mol/L (n = 5) or 5×10^{-6} mol/L (n = 5) was applied topically for 10 minutes. These doses of nifedipine did not affect systemic blood pressure. PAF challenge at 10^{-8} mol/L was implemented after the nifedipine application.

Microvascular permeability. We used fluorescein isothiocyanate–dextran 150,000 mol wt (FITC-Dx 150) as a macromolecular tracer for microvascular permeability. FITC-Dx 150 was administered intravenously as a 100 mg/kg bolus 45 minutes into the stabilization period and followed by continuous infusion (0.15 mg/kg/min) to maintain a constant plasma concentration. Superfusate (5-minute collection samples) and plasma concentrations of FITC-Dx 150 were determined by use of a Perkin-Elmer LS-3 spectrofluorometer (Perkin-Elmer, Norwalk, Conn.). Microvascular plasma clearance was determined from the following formula: Clearance value (nl/min/g) = (Superfusate concentration of FITC-Dx 150 [ng/ml] × Flow rate [ml/min])/(Plasma concentration of FITC-Dx 150 (mg/ml) × Pouch weight). Cumulative clearance values for the complete baseline and experimental periods were computed by simple addition of the 5-minute clearance values.

Arteriolar diameter measurement. We measured arteriolar luminal diameter as the width of the transilluminated blood column. Three to five arterioles (20 to 40 μm in diameter) per animal were studied. The selected vessels were video recorded during the control period and at 2-minute intervals for 10 minutes after the application of PAF. Control diameters were normalized to a value of one, and the experimental diameter was expressed as a ratio of the control diameter (experimental/control = relative luminal diameter). Because topical application of nifedipine caused vasodilation, the arteriolar diameter measured at the end of the administration of nifedipine was used as the baseline to evaluate the effect of subsequent PAF-induced vasoconstriction.

Chemicals. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. PAF was initially dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10^{-2} mol/L and was diluted subsequently to the desired concentrations with a

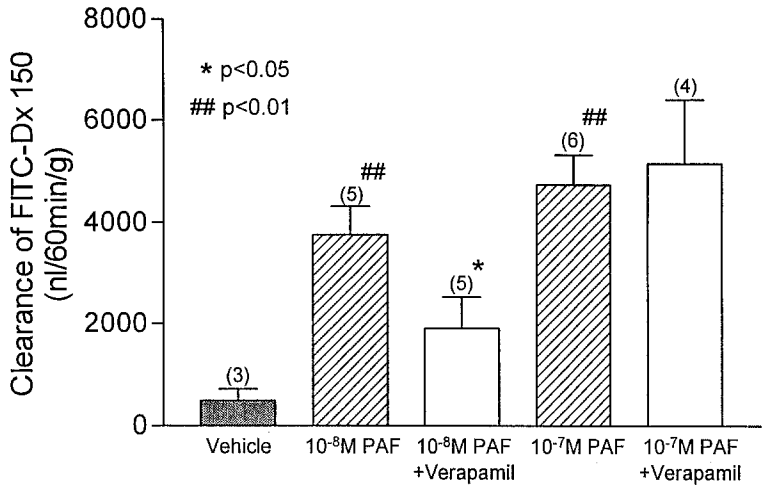

Fig. 3. Statistical analysis of effect of verapamil on PAF-induced increase in FITC-Dx 150 clearance. Data are presented as mean ± SE. *Numbers in parentheses* show number of animals. $*p < 0.05$ compared with 10^{-8} mol/L PAF. $\#\# p < 0.01$ compared with vehicle.

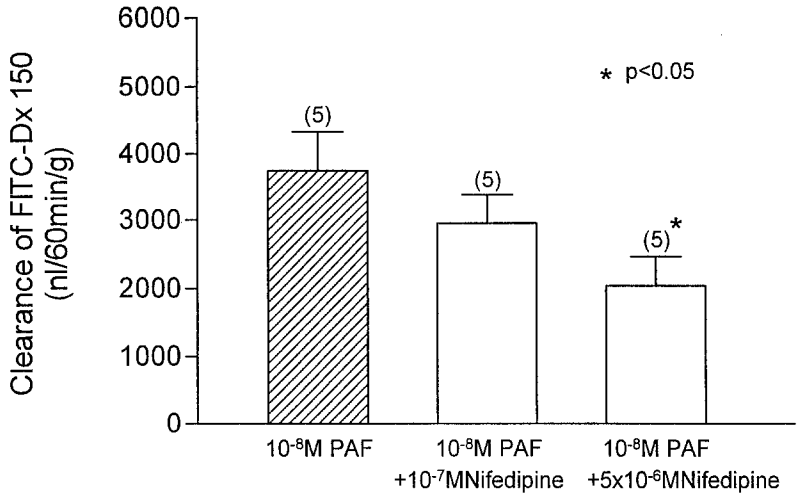

Fig. 4. Statistical analysis of effect of nifedipine on PAF-induced increase in FITC-Dx 150 clearance. Data are presented as mean ± SE. *Numbers in parentheses* show number of animals. $*p < 0.05$ compared with 10^{-8} mol/L PAF.

mixture of 1.5% bovine serum albumin and bicarbonate buffer. Verapamil was dissolved and diluted with saline solution before application. Nifedipine was dissolved in DMSO to 10^{-1} mol/L and was diluted with bicarbonate buffer. The final concentration of DMSO was less than 0.1% and did not affect microvascular parameters.

Statistical analysis. All data are expressed as means ± SE. Date were analyzed by use of unpaired *t* testing and one-way analysis of variance testing followed by Student-Newman-Keuls' multiple range testing. The *p* values less than 0.05 were considered significant.

RESULTS

Initially, both verapamil and nifedipine were designed to be applied topically to the hamster cheek pouch. In preliminary experiments, however, topical application of verapamil at 10^{-5} mol/L caused a significant increase in FITC-Dx 150 clearance (data not shown). Therefore verapamil at 2 mg/kg was applied intravenously. This dose of

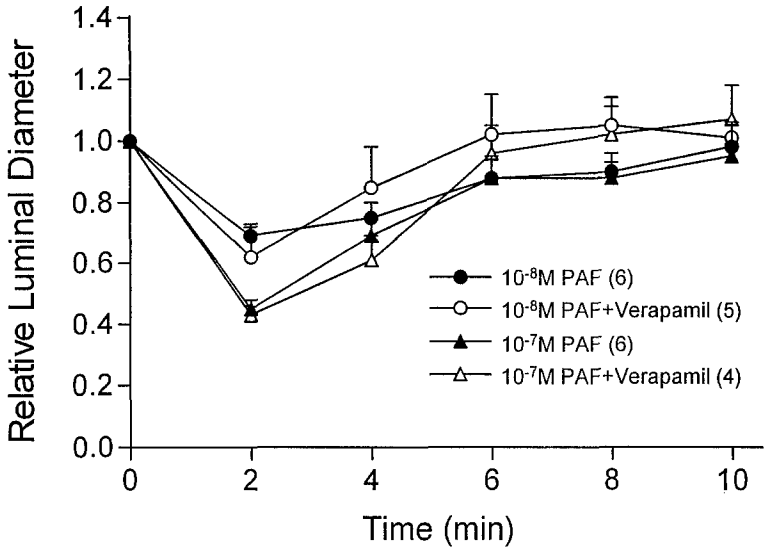

Fig. 5. Effect of verapamil on PAF-induced arteriolar vasoconstriction. Arteriolar diameters were measured during control period and at 2-minute intervals after application of PAF. Control diameters were normalized to value of one, and experimental diameters are presented as ratio of control diameter (experimental/control = relative luminal diameter). *Numbers in parentheses* show number of animals. Data are presented as mean ± SE.

verapamil is efficacious in attenuating macromolecular leakage in IR.[15] Neither intravenous application of verapamil nor topical application of nifedipine caused significant increase in FITC-Dx 150 compared with control.

Permeability

Previous studies in our laboratory have established that PAF at 10^{-7} mol/L causes maximal increase in FITC-Dx 150 clearance in the hamster cheek pouch.[7] In this study we initially chose 10^{-7} PAF ($n = 6$) as the test concentration. We found out experimentally that the effects of this maximal concentration of PAF were not attenuated by verapamil, possibly as a result of stoichiometric relations between agonist and antagonist. Therefore we chose to use PAF at 10^{-8} mol/L ($n = 5$) as the test challenge and applied only this dose to the study of the effects of nifedipine. Both 10^{-8} mol/L and 10^{-7} mol/L PAF caused significant increase in FITC-Dx 150 clearance compared with vehicle (3841.6 ± 260.9 and 4747.7 ± 584.3 vs 498.7 ± 225.3 nl/60 min/gm, respectively; $p < 0.01$) (Fig. 3).

Verapamil. Fig. 3 shows that pretreatment with verapamil significantly attenuated the increase in FITC-Dx 150 clearance induced by 10^{-8} mol/L PAF (1909.1 ± 620.2 nl/60 min/gm, $p < 0.05$). The permeability increase induced by 10^{-7} mol/L PAF was not attenuated by intravenously administered verapamil (5171.7 ± 1238.1 nl/60 min/gm).

Nifedipine. Topical pretreatment with nifedipine attenuated the increase in FITC-Dx 150 clearance induced by 10^{-8} mol/L PAF in a dose-dependent manner. Nifedipine at 5×10^{-6} mol/L significantly reduced the increase in clearance induced by PAF at 10^{-8} mol/L (2037.2 ± 427.5 nl/60 min/gm, $p < 0.05$). Nifedipine at 10^{-7} mol/L also attenuated the 10^{-8} mol/L PAF-induced permeability change (2945.3 ± 437.3 nl/60 min/gm). Nifedipine at 10^{-7} mol/L may represent a near-threshold concentration because the reduction in clearance was not significantly different compared with 10^{-8} mol/L PAF (Fig. 4).

Arteriolar diameter

Topical application of PAF at 10^{-8} mol/L or 10^{-7} mol/L caused maximal vasoconstriction within 2 minutes after PAF application (relative luminal diameter ratios 0.69 ± 0.04 and 0.45 ± 0.03, respectively). Thereafter, arteriolar diameter gradually returned toward the baseline in the 10-minute observation period.

Verapamil. Pretreatment with verapamil did not attenuate the maximal arteriolar constriction at 2 minutes after the application of either 10^{-8} mol/L or 10^{-7} mol/L PAF (0.62 ± 0.10 vs 0.69 ± 0.04 and 0.43 ± 0.03 vs 0.45 ± 0.03, respectively). Verap-

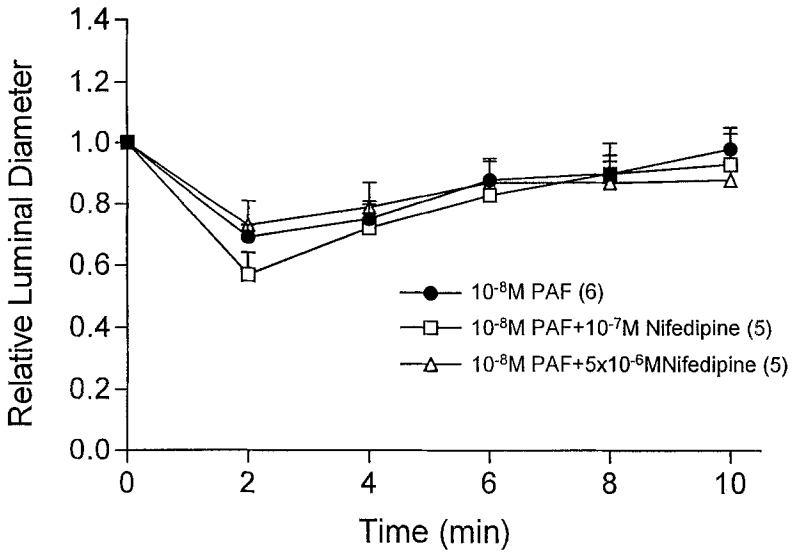

Fig. 6. Effect of nifedipine on 10^{-8} mol/L PAF-induced vasoconstriction. Arteriolar diameters were measured during control period and at 2-minute intervals after application of PAF. Control diameters were normalized to value of one, and experimental diameters are presented as ratio of control diameter (experimental/control = relative luminal diameter). *Numbers in parentheses* show number of animals. Data are presented as mean ± SE.

amil pretreatment had no significant effect on the PAF-induced arteriolar constriction (Fig. 5).

Nifedipine. Nifedipine, at either 10^{-7} mol/L or 5×10^{-6} mol/L, did not reduce the vasoconstriction occurring at 2 minutes after 10^{-8} mol/L PAF challenge (0.57 ± 0.07 and 0.73 ± 0.08 vs 0.69 ± 0.04, respectively). Nifedipine had no significant effect on the vasoconstriction induced by 10^{-8} mol/L PAF (Fig. 6).

DISCUSSION

This study demonstrates that pretreatment with two L-type calcium channel blockers, verapamil and nifedipine, significantly attenuated the increase in FITC-Dx 150 clearance induced by PAF. Neither of the calcium channel blockers affected the PAF-elicited arteriolar constriction.

Attenuation of PAF-induced hyperpermeability by verapamil and nifedipine. In general, the mechanism of increasing macromolecular permeability induced by PAF has been considered to be the result of a wider intercellular gap as a consequence of endothelial cell contraction.[16] PAF causes a reversible change of endothelial cell shape and an increase of albumin transfer across endothelial cell monolayers.[17] Along with $[Ca^{2+}]_i$ increases, actin and myosin may play a central role in the regulation of the width of endothelial intercellular gaps.[18] In in vitro studies, PAF induces a rapid increase of $[Ca^{2+}]_i$ in cultured endothelial cells.[19,20] Furthermore, both PAF synthesis and rapid endothelial cell–dependent polymorphonuclear leukocytes adhesion require Ca^{2+}.[21] Thus an elevation in $[Ca^{2+}]_i$ seems to be one of the initial important events in the cellular signaling cascade associated with stimulation by PAF, as well as other mediators of inflammation.

At least two mechanisms are involved in PAF-induced $[Ca^{2+}]_i$ increase. One is Ca^{2+} influx into the cytoplasm and the other is mobilization of Ca^{2+} from intracellular stores in response to elevated IP_3 through activation of the PLC pathway.[2,12] Of the total increase of $[Ca^{2+}]_i$ caused by PAF, about one fourth is due to the IP_3-induced mobilization of Ca^{2+}.[12] Thus PAF-induced $[Ca^{2+}]_i$ elevation appears to be predominantly due to Ca^{2+} influx; however, the precise pathways through which Ca^{2+} enters into the cytoplasm have not been completely elucidated yet. In platelets, receptor-operated channels have been proposed as a Ca^{2+} entry stimulated by PAF, because voltage-operated channels do not exist in platelets.[22] Recently, direct measurements of Ca^{2+} channel current and $[Ca^{2+}]_i$ have been achieved by use of both patch clamp techniques and fluorochrome-preloaded cells. In cultured endothelial cells, experimental electrophysiologic evidence indicates a lack of functional voltage-operated Ca^{2+} channels; however, there are concerns about the technical difficulties involved in measuring small Ca^{2+} currents, the loss of

Ca^{2+} channels during enzymatic digestion, lack of expression of Ca^{2+} channels, and lack of sensitivity to the normal Ca^{2+} channel agonists and antagonists under the experimental conditions.[23] Nonetheless, with fura-2–preloaded vascular endothelial cells, a new voltage-operated calcium channel (R-type) has been reported in vascular endothelial cells.[24] This R-type channel may play a role in the PAF-elicited cellular signaling events.[24]

Our study shows that blockade of L-type calcium channel inhibits the pathways of PAF cellular signaling in vivo. However, we cannot attribute this property uniquely to a change in the activity of voltage-operated Ca^{2+} channels in endothelial cells. Verapamil inhibits the PAF-elicited responses in platelets[25] and neutrophils[26] by mechanisms other than inhibition of Ca^{2+} influx. In vitro data suggest that PAF receptors are closely linked to Ca^{2+} channels in the plasma membrane.[13,26] This observation is in agreement with the report that specific DHP derivatives are novel PAF-receptor antagonists.[27] The assumed mechanism of action of calcium channel blockers is their binding to a site associated with the PAF receptor-linked channel.[27]

Thus verapamil and nifedipine are likely to modulate the hyperpermeability induced by PAF possibly through inhibition of both Ca^{2+} entry and PAF-receptors. Because neither Ca^{2+} entry antagonist could completely abolish the PAF-induced permeability increase, we postulate that there are other signaling pathways, including PAF-receptors and other Ca^{2+} channels, that participate in the PAF-induced hyperpermeability.

We did not measure leukocyte adhesion to venular endothelium in this study because of logistic reasons. It is unclear whether leukocytes play a role in the PAF-induced hyperpermeability under our experimental conditions. Although some leukocyte adhesion is possibly elicited by 10^{-8} mol/L PAF, maximal leukocyte adhesion is stimulated by PAF at 10^{-9} and 10^{-11} mol/L.[8] It is worth noting that PAF does not stimulate hyperpermeability at these low concentrations.[28] If pretreatment with calcium entry channel blockers leads to priming interactions between PAF and other endogenous agonist, then a more predominant role for leukocytes is possible. We have demonstrated previously that polymorphonuclear leukocytes are involved in mediating the microvascular hyperpermeability caused by priming interactions between PAF and histamine.[29]

L-Type calcium channel blockers and PAF-induced arteriolar vasoconstriction. PAF-induced arteriolar constriction in the hamster cheek pouch is mediated by thromboxane A_2,[6,28] which is generated through phospholipase A_2 activation. In general, within 5 to 10 seconds of PAF challenge, the level of IP_3 increases severalfold, resulting in an increase in $[Ca^{2+}]_i$.[12] Direct measurement of $[Ca^{2+}]_i$ in cultured bovine aortic endothelial cells revealed that 10^{-7} mol/L PAF caused a transient increase of $[Ca^{2+}]_i$, which reached a maximum value within 30 to 60 seconds and subsequently returned to control levels within 9 minutes.[19] The general pattern of the time course of $[Ca^{2+}]_i$ movement seems compatible with that of the PAF-induced arteriolar constriction observed in this study. Generally, application of PAF (at concentrations of 10^{-8} mol/L and higher) to the hamster cheek pouch causes a peak constriction, in a dose-related manner, within the first 2 minutes, which subsequently returns gradually to the base line in about 10 minutes.[6]

The failure of verapamil and nifedipine to inhibit the PAF-induced vasoconstriction may be due to the following reasons: (1) neither verapamil nor nifedipine inhibits phospholipase A_2 activation, and thus do not prevent the production of thromboxane A_2; (2) neither agent prevents the release of Ca^{2+} from intracellular stores through the PLC pathway; (3) either agent may only partially inhibit Ca^{2+} influx; and (4) assuming that verapamil and nifedipine block PAF-receptors, there may be a difference between the PAF receptors present in arterioles and the PAF receptors present in postcapillary venules.[30]

We conclude that L-type calcium blockers may be useful in the management of PAF-induced hyperpermeability but not its associated vasoconstriction. Our data suggest that calcium channel blockade may at least partially protect the microcirculation in disease. Furthermore, PAF-receptor blockade may provide a mechanism for controlling microvascular dysfunction in IR injury.[11]

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DISCUSSION

Dr. Thomas F. Panetta (Brooklyn, N.Y.). Dr. Oshiro et al. have provided data that L-type calcium channel blockers can reduce the capillary leak of macromolecules induced by the topical administration of PAF. However, these calcium channel blockers had no effect on PAF-induced vasoconstriction. There was an eightfold increase in microvascular permeability as a result of the topical application of PAF to the hamster cheek pouch. Pretreatment with topical nifedipine or intravenous verapamil resulted in a 50% decrease in the capillary leak that still remained a fourfold increase over baseline permeability. The Newark group has previously demonstrated a similar effect of verapamil in the ischemic model with rat cremaster muscle. However, in the cremaster muscle model of IR, verapamil maintained the microvascular integrity to near baseline levels.

Why is there an incomplete response to l-type calcium channel blockers in the hamster cheek pouch and a

near-complete response in IR in cremaster muscle? Is this related to differences between topical PAF administration and true IR injury, or is this a function of differences between calcium channel activity in the gracilis muscle versus the hamster cheek pouch? PAF increases intracellular calcium in human and canine endothelial cells, which is not prevented by nifedipine. This response has been demonstrated to be due primarily to R-type calcium channels. Can you speculate on the role of variable responses and the variety of calcium channels including nonspecific cation channels in the sodium-calcium exchanger in both species variations and experimental differences in your findings?

Why does topical verapamil increase macromolecule permeability whereas intravenous administration does not? Although this is an incidental finding that was provided in the manuscript, does this provide any insight into the role of endothelial cells in these interactive cellular processes? Likewise, do differences in results between vasoreactivity and capillary leak reflect a different mechanism or possibly an intact endothelial cell layer interposed between reactive smooth muscle cells at the time of pretreatment?

With ischemia, phospholipase A_2 (PLA_2) activity initiates the membrane release of PAF and arachidonic acid, the precursor of eicosanoids, including thromboxane. Concomitant phospholipase C activation stimulates the inositol pathways to release intracellular calcium stores, which, in combination with protein kinase C activity, opens calcium channels for calcium influx. Capillary leaks, on the other hand, occur relatively late in the cascade and are associated more with reperfusion events that activate cyclooxygenase and lipoxygenase pathways and release free radicals.

Keeping in mind that topical application of PAF is different than an IR model, where does PAF-induced microvascular dysfunction fit into this cascade? Does activation of PAF receptors directly increase phospholipase C activity, or are there changes mediated by PAF-induced increase in eicosanoids, free radicals, or cytokines? Do you think that PAF receptor antagonists might completely abolish the permeability changes caused by exogenous PAF? Are you considering experiments with PAF antagonists and, additionally, with deacylated PAF or lyso-PAF or other PAF analogs to confirm that your findings are both PAF and PAF receptor specific?

In the manuscript there is no mention of intracellular receptors that exist for PAF. I have some concerns that, although topical application of PAF is an excellent means of elucidating some of these complicated biochemical interactions, this model does not result in intracellular increases in PAF, which could potentially initiate alternative cell signaling systems, including second messenger and third messenger cell signaling pathways. Would you comment?

What is the clinical relevance of these findings? Considering the findings, do l-type calcium channel blockers have a role in treating IR disorders or might they be more useful in conditions with severe capillary leaks such as burns, septic shock, or adult respiratory distress syndrome?

Dr. Hidemi Oshiro. To answer your first and third questions, I want to review briefly the PAF-induced signaling pathways. PAF operates through specific receptors on the plasma membrane. These receptors activate phospholipases A_2, C, and D (PLC, PLD), as well as calcium channels. Stimulation of PLC and PLD leads to the activation of protein kinase C (PKC) and of calcium mobilization from intracellular stores (via inositol trisphosphate). Our laboratory has reported that activation of PKC increases microvascular permeability to macromolecules (*Am J Physiol* 1994;266:H1214-20). The activation of PKC may be independent of the action of PAF on calcium entry via calcium channels, which is believed to be the predominant route for calcium-related increases in microvascular permeability. Thus blockade of L-type and R-type calcium channels would be expected to effect only a partial block of the PAF-induced enhancement of microvascular permeability.

Our laboratory has also demonstrated that PAF-induced vasoconstriction is mediated by thromboxane A_2. Again, this PLA_2-mediated pathway does not depend entirely on calcium entry and explains the maintenance of the PAF-vasoconstrictor effect in the presence of nifedipine and verapamil.

Where does PAF-induced microvascular dysfunction fit in the biochemical cascade? The sequence of hypoxia and reoxygenation causes an increase in PAF release in endothelial cells, so there is some in vitro evidence for the production of PAF under conditions that mimic IR (*Am J Physiol* 1992;263:H956-62). As indicated, we have demonstrated in our in vivo preparation that PAF triggers a well-defined biochemical pathway resulting in increased microvascular permeability. As PAF stimulates PLA_2, PLC and PLD, a constellation of prohyperpermeability factors are set in motion, including calcium mobilization, leukotrienes, nitric oxide, and cytokines.

Precapillary and postcapillary effects of PAF are mediated by different receptors (*Am J Physiol* 1991;261:H1648-52) and potentially different biochemical signaling pathways (*Am J Physiol* 1994;266:H1214-20). We have reported that WEB 2086, a universal PAF antagonist, effectively blocks the adhesion of leukocytes to postcapillary venular endothelium in IR (*Surg Forum* 1992;43:376-8). Experiments with lyso-PAF have demonstrated the receptor specificity for PAF.

As indicated earlier, in our in vivo experiments with exogenously applied PAF, we have demonstrated the PAF-stimulated activation of a biochemical signaling pathway that includes PLA_2, PLC, PLD, PKC, and NO. These findings support the efficacy of this experimental tool to elucidate the role of specific agonists in the control of microvascular function in health and disease.

Regarding your question about the differences between models and animal species, in these experiments we

used as a challenge a PAF concentration of 10^{-8} mol/L, which has been reportedly measured in models of IR in the intestine (*Biochem Biophys Res Commun* 1989;158:353-9). It is possible that verapamil might be more efficacious under conditions of IR. It is also possible that the concentrations of PAF produced in the rat cremaster model are lower than 10^{-8} mol/L. It is also known that rats are less sensitive to PAF than hamsters.

Your final question relates to the clinical relevance of our findings. The clinical use of verapamil and nifedipine is well established in specific settings. Our results suggest they might be useful in the management of IR. However, caution must be exercised because these agents limit the extent of the change in microvascular permeability, but may not be as efficacious in controlling the hemodynamic aspects of reperfusion.

I want to add that PAF-receptor antagonists may provide some additional benefit in the treatment of IR because they block the adhesion of leukocytes to endothelium in postischemic tissue. This beneficial action is achieved whether the PAF-receptor antagonist is administered before ischemia or just before reperfusion (*Surg Forum* 1992;43:376-8). These results are encouraging for further exploration of agents that may assist in the postoperative management of vascular diseases.

Dr. Robert W. Hobson II (Newark, N.J.). One of the difficulties in carrying out basic research is to translate the results into our clinical practice. To answer Dr. Panetta's question regarding clinical relevance of Dr. Oshiro's data, we need to define the severity of skeletal muscle injury in terms of clear clinical endpoints. These well-defined endpoints would allow us to compare data from different hospitals and clinics and would facilitate the translation of basic research into clinical practice.

Thus I want to reiterate a challenge to all of us to define endpoints that will allow us to compare our independent surgical results and to conduct prospective randomized trials on the pharmacologic manipulation of the IR syndrome.

Dr. Marsel Huribal (Buffalo, N.Y.). Use of nitric oxide inhibitor had similar results on your model and PAF-induced microvessel leakage. How do PAF and nitric oxide interregulate?

During ischemia there is a significant amount of endothelial cell dysfunction in which there is a significant amount of vasoactive substances that are produced, of which endothelin is at least 100,000-fold more potent than PAF in vasoconstriction and leukocyte activation and free radical production; it also enhances capillary leakage in itself and goes through several calcium mechanisms as you described. I believe endothelin also induces PAF activation. With this in mind, although you have a lot of potent vasoactive substances, how important is PAF or nitric oxide in ischemia or reperfusion injury?

Some patients who are receiving calcium channel blockers still have development of reperfusion injury. What is the role of l-type calcium signaling in the clinical situation?

Dr. Oshiro. We have in vivo evidence demonstrating that the mechanism of PAF-induced increase in permeability involves a stimulated biochemical signaling pathway that includes enhanced activity of nitric oxide synthase (NOS) and increased production of nitric oxide (*Microvasc Res* Sept/Oct. 1995;50:223-34). Our experiments are consistent with the activation of the constitutive form of NOS. We have experimental evidence that NOS activity is elevated after activation of PKC. The experiments reported by Dr. Noel investigate the priming interactions between PAF and histamine. Dr. Noel's studies demonstrated that NO is a factor involved in the priming interactions leading to an increase in microvascular permeability to macromolecules. Whether NO is stimulated by PKC under priming conditions remains to be elucidated.

Endothelins are agents that produce vasopressor responses in whole animals and vasoconstriction in perfused vessels in the range of 10^{-9} mol/L. At lower concentrations, endothelins tend to produce vasodilation. The vasopressor responses to endothelins are concentration and receptor-type dependent. Interestingly and somewhat paradoxically, removal of endothelin-1 (by molecular biology techniques) in mice results in blood pressure higher than normal (*Nature* 1994;368:703-10). PAF is a vasoconstrictor at concentrations of 10^{-8} mol/L and 10^{-7} mol/L, whereas at concentrations lower than 10^{-9} mol/L, it tends to be a vasodilator. We have not yet investigated the possible relations between these clearly relevant and important molecules.